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Technical note

Evaluating the performance of five up-to-date DNA/RNA co-extraction methods for forensic application



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ABSTRACT

The importance of RNA evidence is growing with new developments in RNA profiling methods and purposes. As forensic samples often can be of small quantity, extraction methods with high yields of both DNA and RNA are desirable. In order to identify the optimal DNA/RNA co-extraction workflow for forensic samples, we evaluated the performance of three frequently-used methods, two new approaches for DNA/RNA co-extraction and a manual phenol/chloroform RNA-only extraction method on blood and saliva samples. Based on a comprehensive analysis of the RNA and DNA quantities, as well as the STR genotyping and mRNA profiling results, we conclude that the two frequently-used co-extraction methods, combining commercially available DNA and RNA extraction kits, achieved the best performance. However, not any combination of commercially available DNA and RNA extraction kits works well and extensive optimization is necessary, as seen in the poor results of the two new approaches.

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1. Introduction

With the rapid development of transcriptome analysis methods, the importance of RNA evidence has been recognized over the last decades and an increasing number of studies have shown the potential of RNA profiling for forensic applications. RNA markers can be applied to achieve different purposes, such as body fluid identification [1–4], organ tissue inference [5,6], estimating the age of a stain (time since deposition TsD) [7–9] and estimating the age of the stain donor [10,11]. So far, the most advanced field of application is the identification of body fluids using mRNA profiling, with conventional RT-PCR/CE methods [12–16], or up-to-date massively parallel sequencing (MPS) approaches [17–19]. However, due to the easily degrading nature of RNA molecules, successful RNA profiling usually requires the RNA extracts to have relatively high quantity and quality. Since forensic samples are often low-template and degraded, an effective DNA/RNA co-extraction method with satisfactory DNA/RNA yields and integrity is of great value in practical use.

There are already some commercial DNA/RNA co-extraction kits available, but they were not specifically developed for low-template samples. Hence, some alternative co-extraction methods aimed for criminal casework samples were proposed by several forensic laboratories [16,20,21]. However, only a few comparative studies had evaluated the performance of different RNA-only extraction methods or DNA/RNA co-extraction methods for forensic applications. Grabmüller et al. [22] compared the performance of five commonly used RNA extraction methods, including some commercially available kits on dried blood, liquid saliva, semen and buccal mucosa samples. The different methods exhibited considerable variance concerning RNA and DNA yields, RNA quality and expression levels, and STR profiling success. They concluded that there was no 'best' method to satisfy all demands for co-analysis of RNA and DNA since each method has specific merits and flaws. Loureiro et al. [23] compared two DNA/RNA co-extraction methods on semen samples, a commercial kit that uses a spin mini column methodology, and a quick, simple nucleic acid isopropanol precipitation based protocol. They found that the manual protocol performed worse, probably due to contaminant carryover that inhibits PCR. Bamberg et al. compared four different DNA/RNA co-extraction and re-extraction methods [15]. Best results were achieved with an automated re-extraction method. In brief, DNA was extracted with the Maxwell® FSC DNA IQ™ Casework Kit

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(Promega). From the residue samples, RNA was re-extracted with the Maxwell® 16 RSC miRNA Tissue Kit (Promega). In a recent collaborative exercise, some self-optimized DNA/RNA co-extraction procedures have been established and applied by several forensic laboratories [24].

The aim of this study was to identify optimal DNA/RNA co-extraction methods for forensic samples from three frequently-used methods that have already been applied by several forensic laboratories and two new approaches. We have chosen two co-extraction methods previously described by the Netherlands Forensic Institute (NFI, E1) and the Institute of Environmental Science and Research (ESR, E2) [16,21], as well as the AllPrep DNA/RNA Mini kit (Qiagen, E3). In addition, we tested two in-house approaches: Casework direct kit followed by ReliaPrep™ RNA Cell Miniprep System (E4) and ReliaPrep™ RNA Cell Miniprep System followed by QIAamp DNA Mini Kit (E5). The RNA profiling performance was assessed and compared to the results of a manual phenol/chloroform RNA-only extraction method (E6) [25], which from our experience provides best RNA yields. In addition, we evaluated the RNA and DNA quantities, and the STR genotyping performance.

2. Methods

The experiments were performed by two laboratories, Oslo University Hospital (Laboratory A) and the Zurich Institute of Forensic Medicine (Laboratory B).

2.1. Sample preparation

Human biological samples were collected at each laboratory from three unrelated healthy volunteers after receiving informed consents, respectively. The sampling was performed in agreement with the local ethics regulations (Norway) and approved by the local ethics commission's declaration of no objection (KEK-No. 24-2015, Switzerland). Peripheral blood was collected from finger pricks, saliva was collected by spitting. Subsequently, 2 µL and 20 µL of blood/saliva was pipetted onto swabs and dried at room temperature for at least 12 h. At Laboratory A the tips of the swab were cut with sterile scissors and placed in extraction tubes. The blood tips were stored at room temperature until the extraction procedure, while saliva tips were stored at 4°C. At Laboratory B the swabs were stored at room temperature until further processing.

2.2. Extraction methods

In total, six extraction methods were tested. Methods E1, E2, E3 and E4 were evaluated by Laboratory A, and methods E1, E3, E5 and E6 were evaluated by Laboratory B. An overview on the applied methods is depicted in Fig. 1. One duplicate of 2 µL and 20 µL from each body fluid and each participant was analyzed, summing up to a total of 12 samples per extraction method. Negative controls were also tested. DNA extracts were stored at -20 °C and RNA extracts were stored at -80 °C until further processing.

2.2.1. Extraction method E1: QIAamp DNA Mini Kit (Qiagen) and mirVana™ miRNA Isolation Kit (ThermoFisher)

Co-extraction of RNA and DNA was performed as described by Lindenbergh et al. [16], with small alterations as suggested by the NFI (personal communication): Initially, samples were incubated with the mirVana Lysis/Binding Buffer at 85 °C for 10 min at 750 rpm and cooled down. Proteinase K was added, followed by incubation at 56 °C for 2 h. Absolute ethanol (112.5 µL, 1/4 vol Lysis/Binding buffer) was added for separating the fractions. Then, 48 µL miRNA homogenate was added to the flow through before further RNA purification and addition of 132 µL nuclease free water as the first step of the

RNA purification. The final elution volumes were 60 µL for RNA and 100/40 µL (Laboratory A/B) for DNA, respectively.

2.2.2. Extraction method E2: DNA IQ™ System and ReliaPrep™ RNA Cell Miniprep System (both from Promega)

Samples were processed as described previously [21] according to the DNA IQ™ System—Small Sample Casework Protocol, with preprocessing using Lysis Buffer. Lysis Buffer (250 µL) was added to the samples and incubated at 70 °C for 30 min. Supernatant containing RNA was removed after the first incubation on the magnetic stand, and transferred into a new tube for further purification. Lysis Buffer (100 µL) was added to the DNA fraction and the samples were removed from the magnetic stand and stored at 4 °C until further processing. RNA purification was performed as described in the manufacturer's protocol, with 84 µL Isopropanol added to the lysate (250 µL). The RNA elution volume was 30 µL. The DNA samples were retrieved from storage and quickly vortexed, before placing back on the magnetic stand. Further purification was performed according to the protocol with a DNA elution volume of 100 µL.

2.2.3. Extraction method E3: AllPrep™ DNA/RNA Mini kit (Qiagen)

Initially, samples were lysed with RTL buffer (350 µL) and incubated at 56 °C for 1 h. Lysates were then transferred into new tubes with QIAshredder spin-columns to get rid of the cotton. Further DNA/RNA co-extraction was performed according to the manufacturer's instruction. The elution volumes for RNA and DNA were 30 µL and 100 µL in Laboratory A, and 30 and 40 µL in Laboratory B, respectively.

2.2.4. Extraction method E4: Casework Direct Kit and ReliaPrep™ RNA Cell Miniprep System (both from Promega)

This new protocol was tested, as the Casework direct kit is the in-house DNA extraction method in Laboratory A, therefore, an RNA extraction method that could be combined with the in-house method would be favorable. The swabs were transferred to a spin basket and 200 µL Casework Direct Solution was added, followed by incubation at 70 °C for 30 min, shaking was applied for the last 30 s. The samples were centrifuged at maximum speed for 5 min and the spin baskets were then removed and discarded. There is no further purification of DNA in the casework direct protocol. The sample was separated into 2 tubes, 100 µL for RNA and 100 µL for DNA. Isopropanol (34 µL) was added to the 100 µL RNA-lysate and the 134 µL were transferred to ReliaPrep minicolumns for further purification according to the manufacture's instruction. The RNA was eluted in 30 µL nuclease free water.

2.2.5. Extraction method E5: ReliaPrep™ RNA Cell Miniprep System (Promega) followed by QIAamp DNA Mini Kit (Qiagen)

Initially, samples were lysed with BL + TG buffer (250 µL) and incubated at 56 °C for 1 h. Lysates were then mixed with isopropanol (85 µL) and transferred onto ReliaPrep minicolumns. After centrifugation, RNA extraction was performed according to the ReliaPrep protocol. The liquid flow-through was used to extract DNA with the QIAamp DNA Mini Kit. The elution volumes were 30 µL and 40 µL for RNA and DNA, respectively.

2.2.6. Extraction method E6: manual phenol/chloroform RNA extraction method

RNA extraction with phenol/chloroform was performed as previously described by Juusola and Ballantyne [26] with a minor modification. Initially, samples were lysed with denaturing solution (500 µL) and incubated at 56 °C for 1 h. Lysates were then transferred into new tubes with QIAshredder spin-columns to get rid of the cotton. The elution volume for RNA was 20 µL.

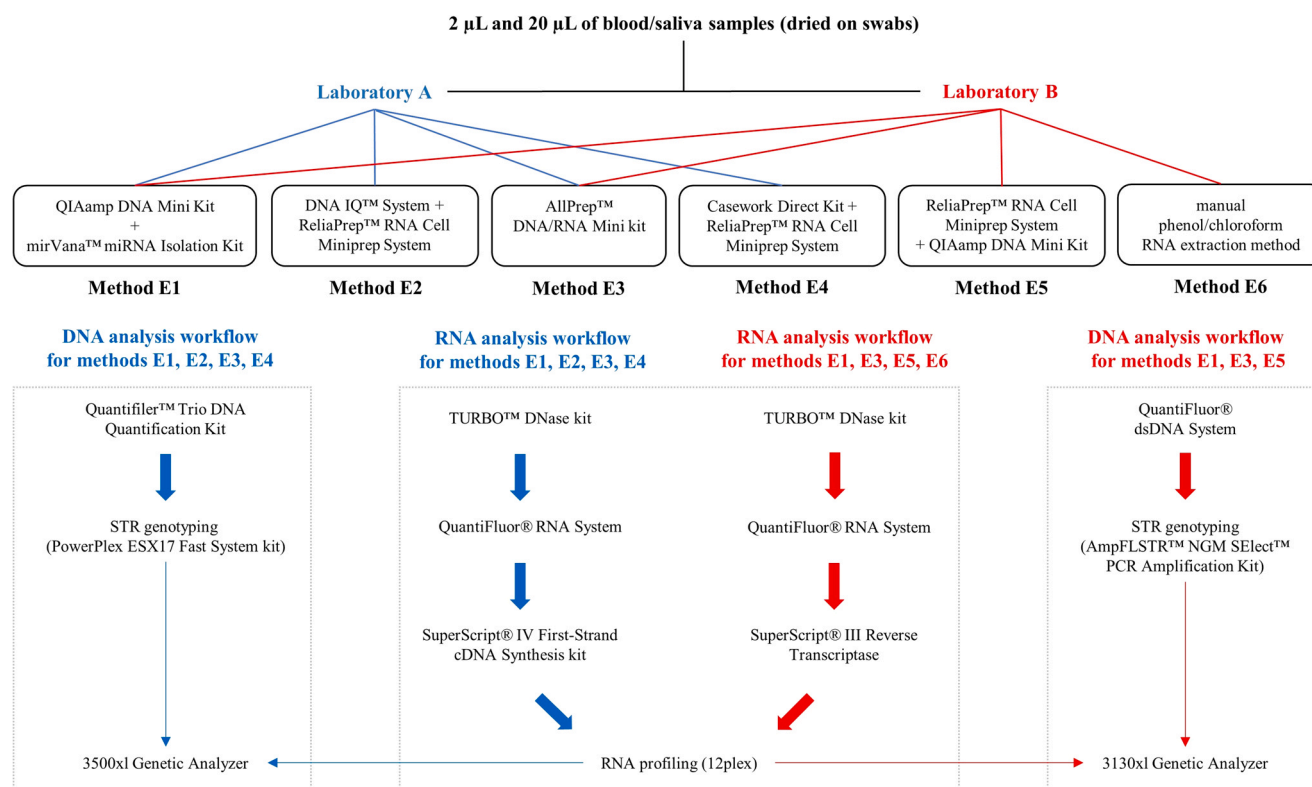


Fig. 1. Schematic representation of the DNA/RNA co-extraction and following analysis procedures.

2.3. DNA analysis

In Laboratory A, DNA samples were quantified with the Quantifiler™ Trio DNA Quantification Kit and the 7500 Real-Time PCR System (ThermoFisher), according to the manufacturer's recommendations. DNA samples were amplified (methods E1, E2 and E3) with the PowerPlex ESX17 Fast System kit (Promega) as recommended in the manufacturer's protocol (0.5 ng template, 25 µL reaction volume and 30 amplification cycles). DNA samples from the casework direct method (extraction method E4) were amplified with addition of the Amp Solution (Promega, 2.5 µL for each sample) and PowerPlex ESX17 Fast on the Veriti™ 96-Well Thermal Cycler (ThermoFisher). Samples were injected on the 3500xl Genetic Analyzer (ThermoFisher) at 1.2 kV for 24 s. The results were analyzed using the GeneMapper ID-X Software V1.6 (ThermoFisher).

In Laboratory B, DNA samples were quantified with the QuantiFluor® dsDNA System on the Quantus™ Fluorometer (Promega), according to the manufacturer's recommendations. DNA was amplified with the AmpFLSTR™ NGM SElect™ PCR Amplification Kit (ThermoFisher) according to the manufacturer's protocol (0.4 ng template, 25 µL reaction volume and 30 amplification cycles). Samples were injected on the 3130xl Genetic Analyzer at 1.2 kV for 18 s. The results were analyzed using the GeneMapper ID-X Software v1.4 (ThermoFisher).

2.4. RNA analysis

DNase treatment was performed with the TURBO™ DNase kit (ThermoFisher) according to the manufacturer's protocol. RNA was quantified using the QuantiFluor® RNA System on a Quantus™ Fluorometer (Promega) with the low standard according to the manufacturer's recommendations. 5 µL (Laboratory A) or 2 µL (Laboratory B) of the RNA extract was added to the working solution. Reverse Transcription was performed using the SuperScript® IV

First-Strand cDNA Synthesis kit (Laboratory A) and the SuperScript® III Reverse Transcriptase (Laboratory B) (both from ThermoFisher), respectively.

For comparison of the results, an in-house RNA 12plex for body fluid identification was used with 2.5 µL input, on a Veriti™ 96-Well Thermal Cycler or GeneAmp® PCR System 9700 (both from ThermoFisher), in Laboratory A and B, respectively. The RNA 12plex includes 12 mRNA markers, 2 human mRNA markers per body fluid (blood, saliva, semen, vaginal secretion, menstrual blood) and 2 bacterial RNA markers (Lcris, Lgas) for the identification of vaginal secretion (Fig. 2). However, only the saliva markers (HTN3, MUC7) and the blood markers (HBA, ALAS2) are relevant here. The RNA 12plex was provided by Laboratory B for all experiments to ensure comparability of results.

Post PCR purification was performed with the Performa DTR Gel Filtration Cartridge (EdgeBio) for all samples analyzed in Laboratory A. Product separation was performed on the Genetic Analyzers 3500xl (Laboratory A) or 3130xl (Laboratory B) (both from ThermoFisher), with 1 µL template and 10 s injection at 3 kV, 60 °C (Laboratory A) or 1 µL template and 10 s injection at 1.2 kV, 60 °C (Laboratory B).

3. Results

3.1. DNA results

DNA quantity of the samples from Laboratory A ranged from 0.81 to 946 ng (mean 121), while DNA quantity of the samples from Laboratory B ranged from 2.0 to 190 ng (mean 23.5). The average DNA quantity differed among extraction methods (Fig. 3). For Laboratory A, the lowest average DNA quantity was from method E3 (54.8 ng), while the highest average was from method E1 (252 ng). For Laboratory B, the lowest average quantity was observed from method E5 (14.1 ng), while the highest average quantity came from

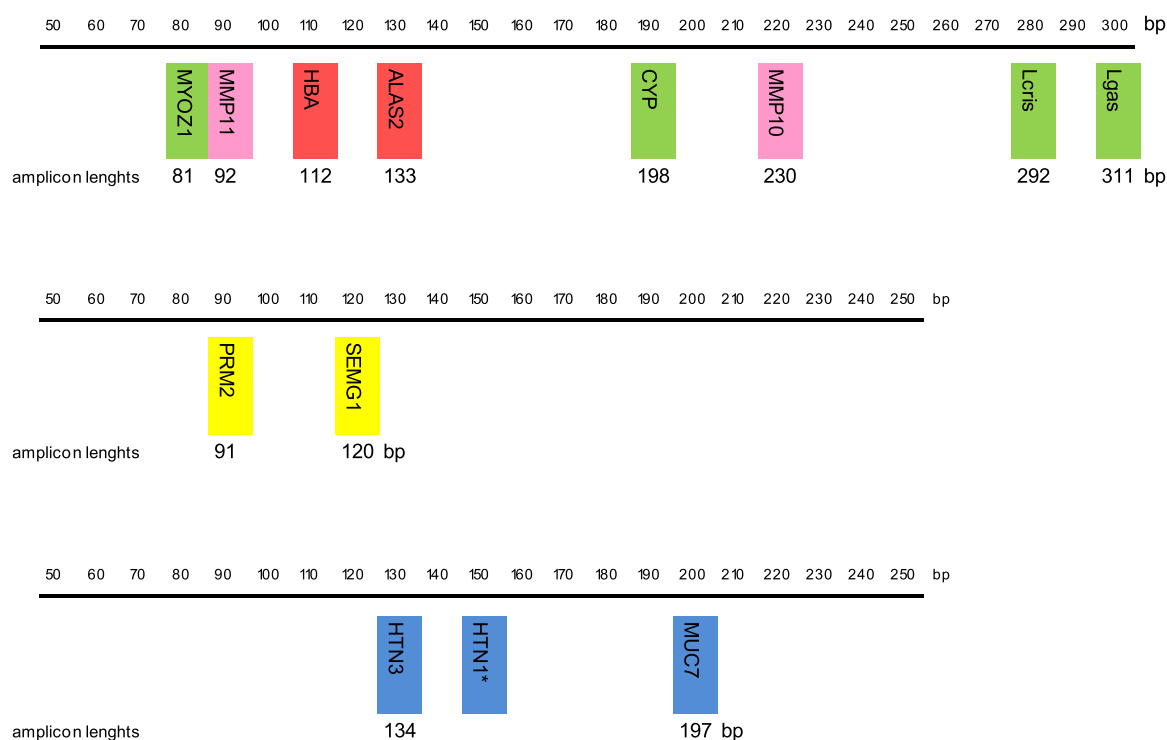


Fig. 2. In-house RNA 12plex, including 2 markers per body fluid (blood (red), saliva (blue), semen (yellow), vaginal secretion (green), menstrual blood (pink), plus 2 microbial vaginal markers (green)). The amplicon lengths are depicted, the fluorescent dyes are FAM, VIC, NED, from top to bottom. *HTN1 is an isoform that is also recognized with the HTN3 primer pair. (For interpretation of the references to color in this figure, the reader is referred to the online version of this article.)

method E1 (35.9 ng) (Fig. 3). Subsequent DNA genotyping analysis showed satisfactory results, full DNA profiles of good quality were achieved from all extraction methods, except for method E5.

3.2. RNA results

3.2.1. RNA yield

For Laboratory A, the measured RNA quantity ranged from 2.64 to 180 ng (mean 26.0). For Laboratory B, the measured RNA quantity ranged from 10.2 to 483 ng (mean 155). The average quantity differed among laboratories and methods (Fig. 4). For both laboratories, the lowest average quantity was from method E3 (6.62 ng for Laboratory A and 19 ng for Laboratory B), while the highest average was from method E1 (50.8 ng for Laboratory A and 336 ng for Laboratory B). No quantitation results exist for the samples extracted by method E4, due to the need for a re-run of the RT-PCR and therefore no sufficient volume for quantitation was available.

3.2.2. mRNA profiling

The Genetic Analyser 3500xl used by Laboratory A has a different sensitivity and maximum peak height than the Genetic Analyser 3130xl used by Laboratory B. For a better comparison between the labs, the peak heights for samples analyzed by Laboratory A were divided by 3 following previously published recommendations [27].

For Laboratory A, all expected peaks corresponding to the correct body fluids were observed from the analysis results of samples extracted by methods E1–E3, while weaker signals were observed from method E4 (Fig. 5). The average peak height differed among the top 3 methods, with the highest observed average from method E2 followed by methods E3 and E1. For Laboratory B, more robust mRNA profiling results (more mRNA markers with higher peak heights) were obtained from methods E1 and E6, irrespective of sample type and input amount. The highest peak was from MUC7, which was

observed in a 20 µL saliva sample extracted with method E1. The poorest mRNA profiling result was observed from method E3, with the lowest number of successfully detected mRNA markers. The measured RNA quantities did not correlate well with the observed peak heights, in general or when considering the 4 mRNA markers separately (Fig. 6). However, the general trend is visible.

4. Discussion

Within this study, we identified expedient DNA/RNA co-extraction methods among three frequently-used and two new approaches in a forensic setting. We tested five different DNA/RNA co-extraction methods and compared the RNA performance to a manual phenol/chloroform RNA-only extraction method. We evaluated the RNA and DNA quantities, as well as STR and RNA profiling performance. Methods E1–E3 and E5 use a waste product during RNA- or DNA-extraction to recover the DNA or RNA, respectively. In method E4 the lysate is divided into 2 aliquots, for DNA and RNA isolation, which is disadvantageous for optimal DNA and RNA yields.

All extraction methods achieved good DNA genotyping results based on the RFU values of detected peaks, except for method E5. Method E1 showed significantly better results than all other methods, when only the DNA quantity is considered. A difference in DNA yield between Laboratory A and B was observed, which is most likely due to the different quantitation methods, fluorescence vs quantitative PCR. The measured quantities are not absolutely accurate for low-template samples, but still a good measure to calibrate the downstream analysis and differences between labs and methods are expected. In addition, the different elution volumes could have an impact on the final DNA yield. It is possible that with the higher elution volume used by laboratory A, more DNA could be recovered from the column membranes. A good DNA genotyping result from a DNA/RNA co-extraction is of high importance in casework, where

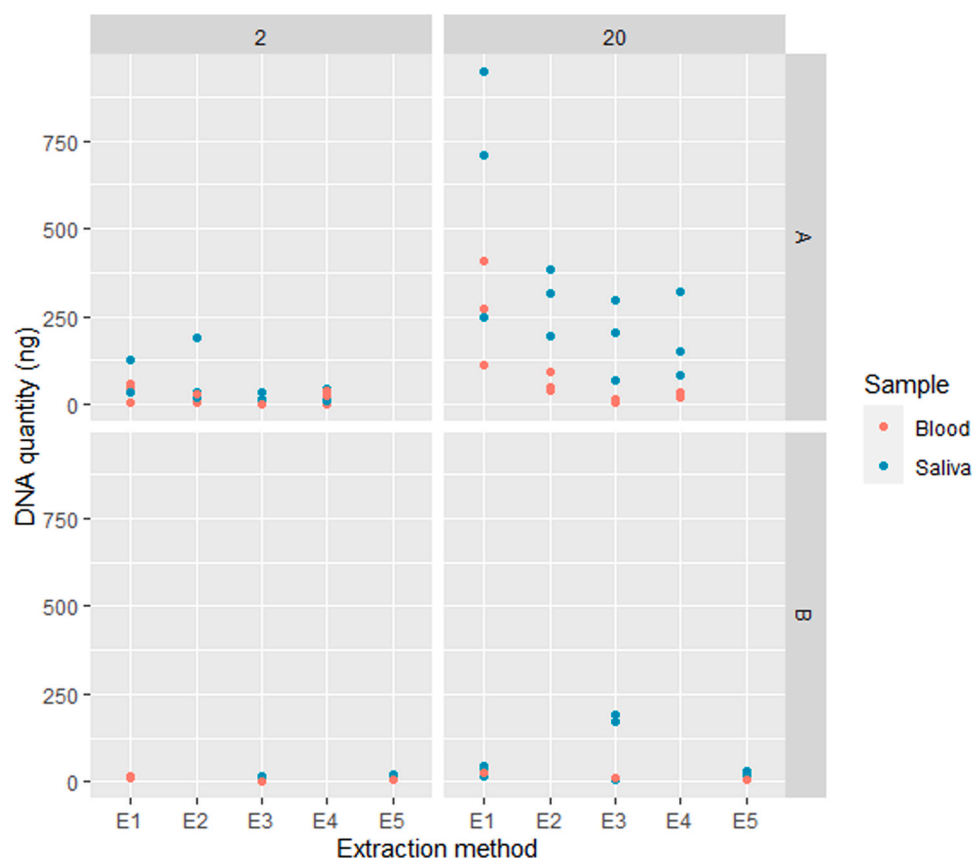


Fig. 3. The measured DNA quantity (ng) in the samples containing 2 µL or 20 µL of blood (red) or saliva (blue), extracted by Laboratory A with methods E1–E4 and Laboratory B with methods E1, E3 and E5, method E6 was an RNA-only extraction method and thus is not exhibited here. (For interpretation of the references to color in this figure, the reader is referred to the online version of this article.)

sample material is limited, and there is often no opportunity for new sample collection, e.g. from swabs. A method that provides equal or better DNA results than existing DNA extraction methods is essential if co-extraction should be prioritized in a case.

Complete RNA profiles (all expected peaks detected) were obtained for all samples extracted with methods E1–E3 from Laboratory A. However, some peaks were not detected (allele drop-out) in RNA profiling results from Laboratory B, especially for the 2 µL saliva samples. This might be simply due to the Genetic Analyzer 3500xl being more sensitive than 3130xl. When comparing the methods in parallel, extraction method E1 showed the best DNA/RNA concentration and profiling results. In addition, method E6 also performed well judged by the RNA yield and peak height, but this method was designed for RNA extraction only, thus could not be the first choice when DNA genotyping is also required. Extraction method E4 was the fastest and the easiest method to perform. Unfortunately, the RNA results were of low quality compared to the other methods. This method has not been tested for RNA extraction previously and it is possible that the extraction buffer conditions are not optimal for RNA isolation. Extraction method E5 was a self-created combination that was not optimized, partly explaining the unsuccessful DNA genotyping results in some samples. Even though, the performance of the ReliaPrep RNA Cell Miniprep System in the RNA extraction part was good.

The RNA quantity did not correlate well with the final RNA profiling result when considering the RFU values of detected markers. However, the general trend was visible. This is probably because the RNA quantitation method is not human specific and especially for saliva samples the presence of bacterial RNA could

influence the quantitation results. Another explanation could be that the expression of different markers varied in the transcriptome of different individuals. Besides, inhibition could play a role in downstream analyses, e.g. from the DNase inactivation reagent in the TURBO™ DNase treatment.

If only considering time and labor, extraction method E3 comes best out of the tests. However, the quantitation results for both RNA and DNA showed an unsatisfactory performance for this method. Extraction methods E1 and E2 are quite similar for several pipetting steps. Considering time of procedure and co-extraction performance, these 2 methods were both practicable when dealing with forensic samples.

There are also some limitations in our current study. We had tested blood and saliva samples, in a reasonable number of samples. However, this is still a limited selection of body fluids and small sample size, considering the huge variation in the DNA and RNA results. We also did not test any automated protocols, which would be advantageous for high throughput analyses. Though, some of the herein manually performed kit based methods could easily be transferred on a robot. Since the performance of RNA profiling was only evaluated based on mRNA markers, we cannot say whether the optimal co-extraction methods identified in this study are also suitable for small RNA isolation. Due to the shorter length, additional enrichment or purification steps are sometimes suggested by commercial RNA extraction kits (e.g. mirVana™ miRNA Isolation Kit) to obtain a better recovery rate of small RNA molecules. Therefore, assessment of these co-extraction methods with other previously proposed small RNA markers for body fluid identification would be worth investigating by further studies focusing on small RNA profiling.

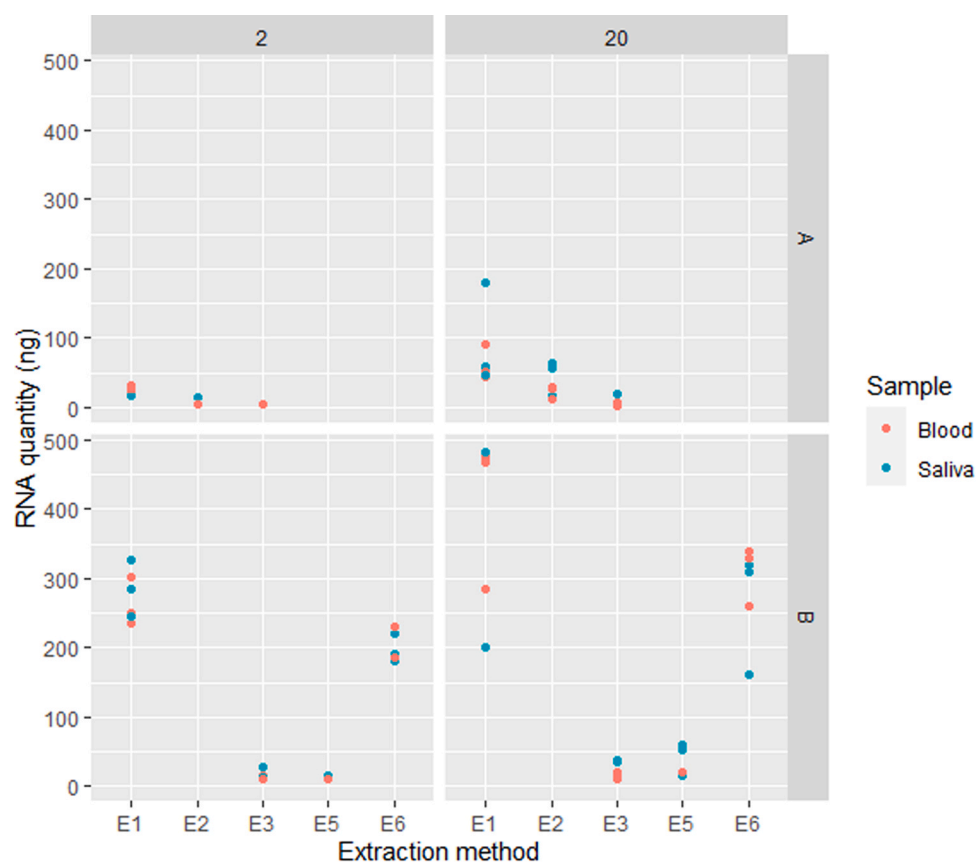


Fig. 4. The measured RNA quantity (ng) in the samples containing 2 µL or 20 µL of blood (red) or saliva (blue), extracted by Laboratory A with methods E1–E3 and Laboratory B with methods E1, E3, E5 and E6. (No quantitation results exist for the samples extracted by method E4). (For interpretation of the references to color in this figure, the reader is referred to the online version of this article.)

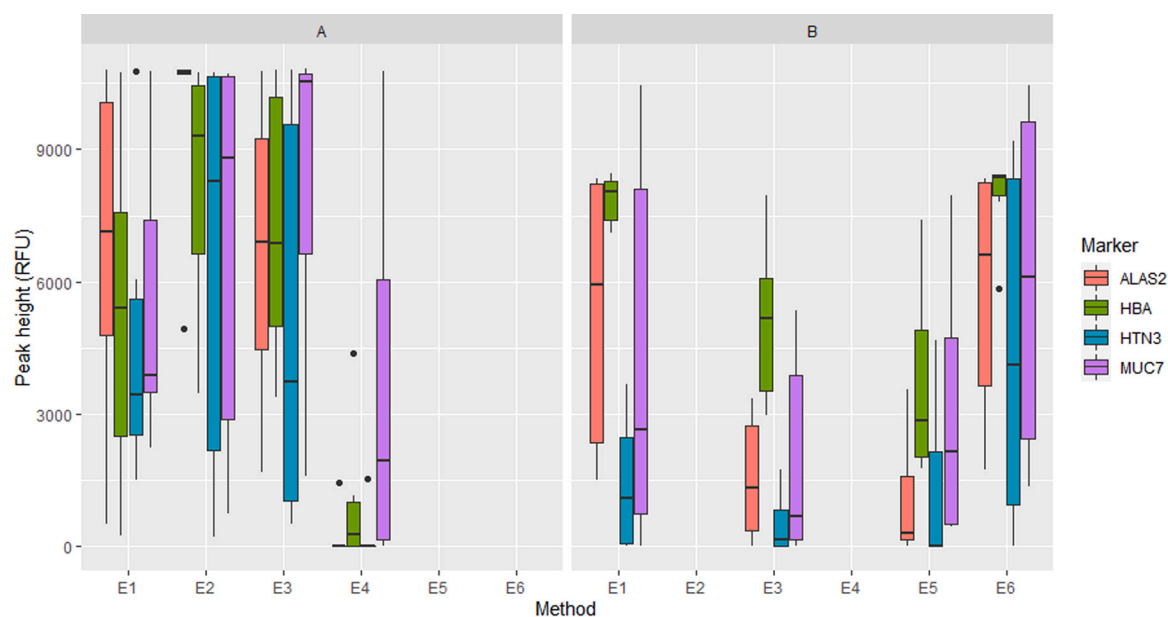


Fig. 5. Boxplot showing the RFU values for the blood (ALAS2 and HBA) and saliva (HTN3 and MUC7) mRNA markers detected in samples extracted with methods E1–E4 by Laboratory A and methods E1, E3, E5 and E6 by Laboratory B.

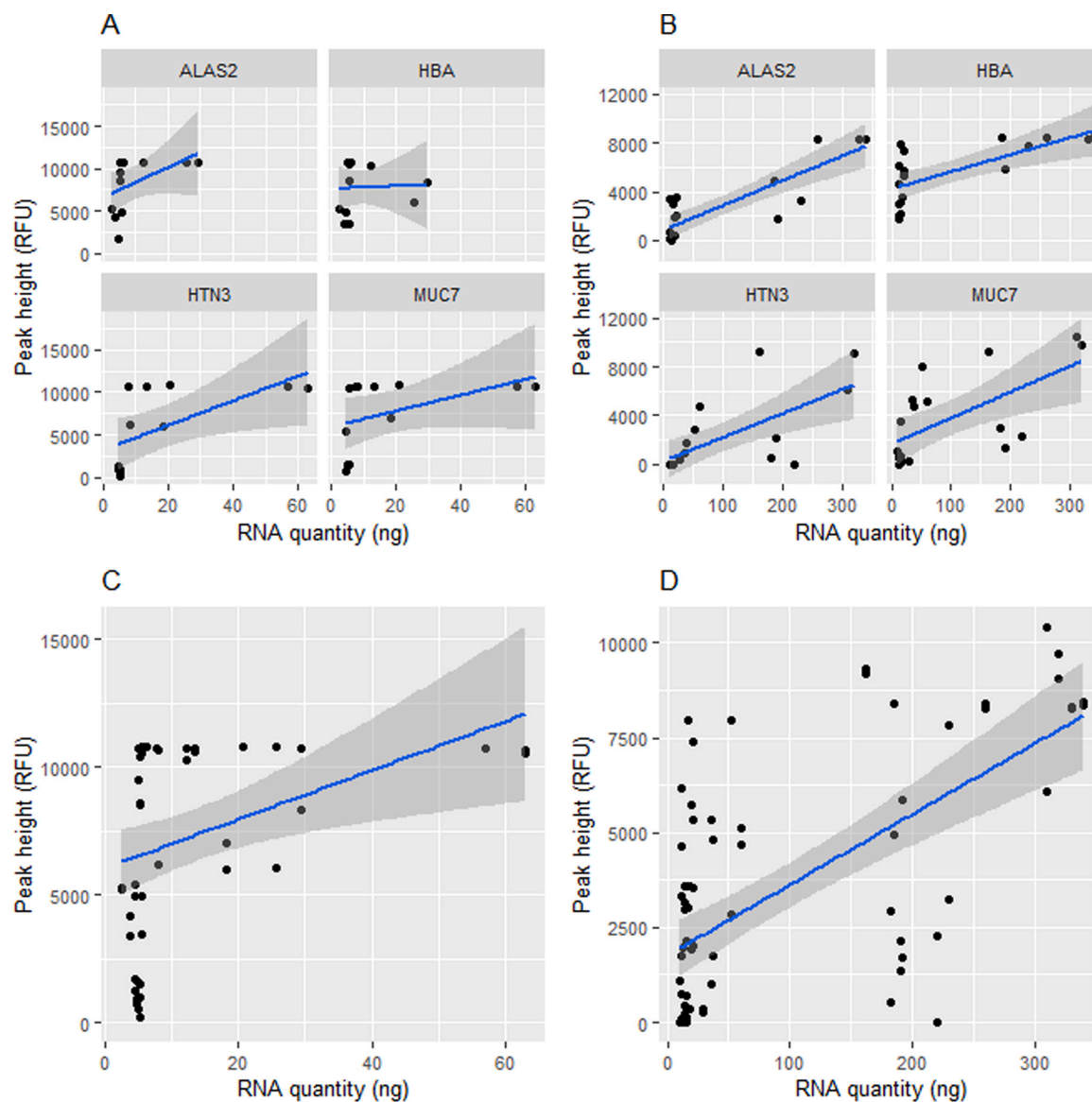


Fig. 6. Peak heights (RFU) plotted against RNA quantity (ng), depicted for the 4 mRNA markers and for the 2 laboratories, separately (A, B) and over all markers and samples (C, D). Laboratory A, $p < 0.005$, $R^2 = 0.16$, Laboratory B, $p < 0.005$, $R^2 = 0.41$ (model based on all data per laboratory).

5. Conclusion

We evaluated the performance of five different DNA/RNA co-extraction methods and compared the RNA profiling results to a manual phenol/chloroform RNA-only extraction method on blood and saliva samples. Variable DNA/RNA quantities were observed among the tested methods. Even with low input blood/saliva samples (2 μ L), successful DNA genotyping was achieved with all methods, except for method E5. The RNA profiling results showed that methods E1 and E2, which are two modified and optimized co-extraction methods combining commercially available DNA and RNA extraction kits, achieved the best performance in this study. However, the suboptimal performance of methods E4 and E5 show that not any combination of commercially available DNA and RNA extraction kits works well and extensive optimization is necessary. In short, our findings could provide instructive information for future studies regarding the selection of DNA/RNA co-extraction methods.

CRediT authorship contribution statement

Shouyu Wang: Investigation, Methodology, Formal analysis, Writing – review & editing. **Gnanagowry Shanthan:** Investigation, Methodology, Writing – review & editing. **Mariam Mjærøum Bouzga:** Conceptualization, Investigation, Writing – review & editing. **Huyen Mong Thi Dinh:** Investigation, Writing – review & editing. **Cordula Haas:** Conceptualization, Investigation, Methodology, Formal analysis, Writing – review & editing. **Ane Elida Fonneløp:** Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2021.110996.

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